——————Original Article——————

ASSOCIATION BETWEEN A pMAH135 PLASMID AND THE PROGRESSION OF PULMONARY DISEASE CAUSED BY *MYCOBACTERIUM AVIUM*

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Abstract [Background] Pulmonary disease caused by nontuberculous mycobacteria has a variable clinical course. Although this is possibly the result of not only host factors, but also bacterial factors, many questions remain to be answered regarding these manifestations. [Methods] To assess the relationship between the progression of pulmonary *Mycobacterium avium* disease and bacterial factors, we performed variable number tandem repeats (VNTR) typing analysis of *M.avium* tandem repeats (MATR) in *M.avium* isolates from 46 patients with different clinical courses, and furthermore, examined the association between disease progression and a pMAH135 plasmid derived from *M.avium*. [Results] In patients whose treatment was initiated because of worsened chest radiograph findings and/or clinical symptoms within 18 months after being diagnosed with pulmonary *M.avium* disease, the detection rate of 6 genes located in pMAH135 was 35.3−47.1% for 17 isolates. However, in untreated patients with a stable condition, these rates were 10.3−13.8% in 29 isolates. MATR-VNTR typing analysis showed that isolates from patients with worsened disease and those with stable disease are clustered differently. In cluster III, the number of isolates from patients with worsened disease was higher than that from patients with stable disease (p＝0.019), and furthermore, the number of isolates carrying pMAH135 genes was higher than that not carrying pMAH135 genes (p≦0.001). [Conclusion] These results indicate an association between the progression of pulmonary *M.avium* disease and pMAH135. The presence of pMAH135 genes might be a useful prognostic indicator for pulmonary *M.avium* disease and may serve as one criterion for treatment initiation.

Key words : *Mycobacterium avium* subsp. *hominissuis*, Disease progression, Variable number tandem repeats, pMAH135 plasmid

Introduction

Nontuberculous mycobacteria (NTM) infection is thought to be caused by NTM residing in the environment, including residential soil or bathrooms[1) 2)], and the prevalence of NTM disease is increasing worldwide. In Japan, the prevalence per 100,000 population has increased greatly from 0.82 in 1971 to 5.9 in 2001[3)]. Today, the rate is estimated to be around 15.0, which is substantially higher than the rates seen in the United States and Europe[4)−6)].

Pulmonary disease caused by NTM is divided into two major disease types, the nodular bronchiectatic type and the fibrocavitary type, with the former being more prevalent[7)−9)]. However, the mechanisms involved in the development and exacerbation of pulmonary NTM disease have yet to be elucidated. Although some patients remain stable without treatment, others have deteriorating symptoms despite drug

therapy, demonstrating the disease's diverse clinical course[8)9)]. Therefore, it would be helpful from the viewpoint of treatment if the clinical course of pulmonary NTM disease could be predicted. The establishment of pulmonary NTM disease or the variable clinical course involves bacterial factors[10)] in addition to host-related risk factors including decreases in the levels of estrogen[11)], a major female sex hormone, or the presence of polymorphisms in *NRAMP1* (encoding natural resistance-associated macrophage protein 1)[12)].

With regard to mycobacterial plasmids, previous studies isolated pVT2[13)] and pLR7[14)] from *M.avium*. Given their relatively small size of 4.8−16 kb and their lack of virulence genes, the significance of these plasmids is currently unknown. Our previous study has shown the presence of a circular plasmid derived from *M.avium* strain TH135, designated pMAH135[15)]. This novel plasmid codes genes that are associated with pathogenicity of mycobacteria and its resistance

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to antimicrobial agents.

In this study, we examined an association between the pMAH135 plasmid and disease progression in a group of patients with different symptoms of pulmonary disease.

Material and Methods

Subjects

We have performed this study using *M.avium* isolates from patients who did not undergo treatment immediately after diagnosis with pulmonary *M.avium* disease in strains used in a previous study[16]. These isolates comprised 46 clinical isolates provided by nine National Hospital Organization hospitals throughout Japan: 5 isolates from Asahikawa Medical Center, 3 from Nishi-Niigata Chuo National Hospital, 15 from Ibarakihigashi National Hospital, 6 from Tenryu Hospital, 3 from Kinki-chuo Chest Medical Center, 8 from Toneyama National Hospital, 1 from Nishibeppu National Hospital, 3 from Fukuoka Higashi Medical Center, and 2 from Minami Kyushu National Hospital. *M.avium* subsp. *hominissuis* strain TH135 was isolated from the sputum of a patient with pulmonary *M.avium* disease at Higashi Nagoya National Hospital of the National Hospital Organization.

Of those who had been diagnosed with pulmonary *M.avium* disease compatible with the diagnostic criteria of the American Thoracic Society and the Infectious Diseases Society of America (ATS/IDSA)[17] between July 2008 and September 2009, patients with few microbiological findings in their sputum specimens or bronchial washes and patients with hardly any subjective symptoms or mild clinical or pulmonary symptoms were followed-up regularly for 18 months. Treatment was initiated if conditions worsened in relation to those at diagnosis during the follow-up period, as judged by exacerbation of chest radiograph findings (including chest computed tomographic images), clinical symptoms and/or microbiological findings, and such patients were categorized into the deteriorated disease group (n=17). Patients in a stable condition were categorized into the stable disease group (n=29). *M.avium* isolates were obtained from the sputa of patients in both groups. Only one strain from each patient was analyzed in this study.

This study was approved by the Ethics Review Committee for Human Research of the Higashi Nagoya National Hospital, and written informed consent was obtained from all patients.

Identification of subspecies of *M.avium*, growth condition, and DNA isolation

The subspecies of *M.avium* clinical isolates was identified as *M.avium* subsp. *hominissuis* by sequence analysis of the 3´ fragment of the *hsp65* gene[18]. The organism was grown in Middlebrook 7H9 liquid medium supplemented with 10 % oleic acid/albumin/dextrose/catalase enrichment (Difco Laboratories, Detroit, MI) at 37°C. DNA was extracted using InstaGene Matrix (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instructions.

Polymerase chain reaction and sequence analysis

The presence of pMAH135 genes in *M.avium* isolates was determined by using specific polymerase chain reaction (PCR) primers, as described previously[15]. The resulting PCR products were purified using a GenElute PCR DNA purification kit (Sigma-Aldrich, St. Louis, MO), and direct sequencing analysis was performed using the same primers as those used for PCR. The resulting nucleotide sequences were compared with the sequence data of pMAH135. The suitability of the present DNA samples for screening clinical isolates by PCR was determined by amplification of the *hsp65* gene—the gene used to identify subspecies of *M.avium* isolates.

Molecular typing

Variable number tandem repeats (VNTR) typing analysis using *M.avium* tandem repeats (MATR) was carried out using 15 VNTR loci (MATR-1 to MATR-16, except for MATR-10) and the corresponding primer set as described previously[19]. After the number of base pairs in the target VNTR loci was estimated by agarose gel electrophoresis in relation to molecular weight markers, the number of repetitions of various VNTR loci in each strain was determined and regarded as an allele profile. The Manhattan distance was determined on the basis of each obtained allele profile, and the genotypic diversity of *M.avium* isolates was analyzed with a Fitch-Margoliash algorithm using the PHYLIP software (version 3.68). The phylogenetic distribution was generated according to the genotypic diversity of isolates, using the FigTree software (version 1.3.1).

Statistical analysis

Fisher's exact test was used for categorical variables and Student's t-test was used for continuous variables. The genetic distances estimated from the Manhattan distance matrix data of *M.avium* isolates were analyzed using a Mann-Whitney comparison test described previously[20]. All statistical analysis was performed using the GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA). P values <0.05 were considered significant.

Results

Characterization of subjects

Of those who had been diagnosed with pulmonary *M.avium* disease compatible with ATS/IDSA diagnostic criteria, patients who had few microbiological findings in their sputum specimens or bronchial washes and patients with hardly any subjective symptoms or mild clinical or pulmonary symptoms did not undergo treatment immediately, but were followed up for 18 months with regular checkups. The patients were subsequently divided into two groups: a deteriorated disease group with 17 patients who had worsened chest radiograph findings, clinical symptoms, and/or microbiological findings during the observation period, and a stable disease group with 29 patients who did not have disease exacerbation.

Age, sex, and clinical characteristics of the subjects are summarized in Table 1. The deteriorated disease group was composed of 7 men and 10 women (mean age 65.4±11.8 years). The stable disease group was composed of 29 persons who were not significantly different from the deteriorated disease group with respect to age (68.1±12.9 years, p＝0.48), sex (9 men and 20 women, p＝0.53), or proportion with underlying disease (deteriorated group, 41.2％; stable group, 51.7％; p＝0.55). In addition, although more subjects had nodular bronchiectatic disease than fibrocavitary disease, there were no significant differences in the radiographic type of disease between the deteriorated disease and stable disease groups (fibrocavitary disease, 7 vs. 10, respectively, p＝0.76; nodular bronchiectatic disease, 9 vs. 17, respectively, p＝0.77).

Screening of clinical isolates for genes located in pMAH135

In the previous study, the complete genome sequence of *M.avium* strain TH135 was determined[21]. Furthermore, we have demonstrated the presence of a circular plasmid, designated pMAH135[15]. This plasmid consists of 194,711 nucleotides and 164 coding sequences (CDSs). Of the pMAH135 CDSs, attention must be paid to those that encode proteins involved in mycobactin biosynthesis[22][23] and the type VII secretion system[24]–[26], both of which are important to mycobacterial virulence as well as to proteins with putative conserved domains of the multidrug efflux transporter[27].

To examine the relationship between the progression of pulmonary *M.avium* disease and pMAH135, we screened 46 clinical isolates provided by nine National Hospital Organization hospitals throughout Japan for 6 CDSs (MAH_p01, MAH_p47, MAH_p49, MAH_p59, MAH_p85, and MAH_p143) located in pMAH135[15]. MAH_p01 encodes the *repA* gene, which is thought to be the replication origin of pMAH135. The remaining 5 CDSs are involved in the pathogenicity of mycobacteria and its resistance to antimicrobial agents[15]. *M.avium* isolates were isolated from the sputa of 17 patients in the deteriorated disease group and 29 patients in the stable disease group. In this study, PCR was carried out to examine the presence of 6 pMAH135 genes using specific primers, yielding PCR products whose size and sequence match those of CDSs located on the pMAH135. As shown in Table 2, pMAH135 genes were found in 35.3−47.1％ of isolates from the deteriorated disease group compared with 10.3−13.8％ of isolates from the stable disease group. In particular, the detection rate of MAH_p47, MAH_p49, and MAH_p143 was significantly higher in isolates from the deteriorated disease group than in those from the stable disease group. These findings show that pMAH135 genes were more prevalent in isolates from the deteriorated disease group than in those from the stable disease group.

Table 1 Demographic and clinical characteristics of patient groups

Characteristic	Deteriorated disease group (n＝17)	Stable disease group (n＝29)	P value
Age, years			
Mean±SD	65.4±11.8	68.1±12.9	0.48
Range	44−84	34−84	
Sex, n (％)			0.53
Male	7 (41.2)	9 (31.0)	
Female	10 (58.8)	20 (69.0)	
Underlying disease, n (％)	7 (41.2)	15 (51.7)	0.55
Type of disease, n (％)			
Fibrocavitary disease	7 (41.2)	10 (34.5)	0.76
Nodular bronchiectatic disease	9 (52.9)	17 (58.6)	0.77
Solitary lung nodule	0 (0)	1 (3.4)	1.0
Other	1 (5.9)	1 (3.4)	1.0

SD, standard deviation

Table 2 Detection rates of pMAH135 genes in *M.avium* isolates from deteriorated and stable pulmonary disease patients

Locus tag	Deteriorated disease patients[a] (n＝17)	Stable disease patients[b] (n＝29)	P value
MAH_p01	7 (41.2％)	4 (13.8％)	0.07
MAH_p47	8 (47.1％)	4 (13.8％)	0.019
MAH_p49	8 (47.1％)	4 (13.8％)	0.019
MAH_p59	6 (35.3％)	3 (10.3％)	0.058
MAH_p85	6 (35.3％)	3 (10.3％)	0.058
MAH_p143	8 (47.1％)	4 (13.8％)	0.019

[a] Strains from the sputa of patients with deteriorated pulmonary disease
[b] Strains from the sputa of patients with stable pulmonary disease

Relation between pulmonary *M. avium* disease progression and pMAH135 based on VNTR genotyping

VNTR typing analysis using MATR of the clinical isolates from patients in both groups was performed to examine the relationship between the progression of pulmonary *M. avium* disease and VNTR genotype. MATR-VNTR analysis is a rapid and highly discriminating subtyping method for *M. avium*[19]. The tandem repeat loci or repetitive units used in VNTR typing analysis are interspersed throughout chromosomes with multiple sequences of 10- to 100-bp minisatellites. The number of repetitions in the sequence is known to vary specifically among bacterial strains and thus allows for their discrimination[28].

As shown in Fig. 1, the *M. avium* isolates examined in this study were classified roughly into three clusters: cluster I, cluster II, and cluster III (including strain TH135). Clusters I, II, and III accounted for 17.6% (3/17), 29.4% (5/17), and 52.9% (9/17) of the isolates from the deteriorated disease group (Table 3), respectively, showing that the proportion of cluster III isolates was significantly larger than that from the stable disease group ($p = 0.019$). Furthermore, to examine relationships between VNTR genotype and disease progression in pulmonary *M. avium* disease, we compared the genetic distances of clinical isolates from the deteriorated and the stable disease group as previously described[20]. *M. avium* strain TH135 was used as a reference to evaluate the Manhattan distance of each *M. avium* isolate. Among the 46 *M. avium* isolates, the genetic distance of each clinical isolate from the reference strain was associated with the highest likelihood of disease progression (subjects with deteriorated disease vs. subjects with stable disease, $p = 0.035$; Fig. 2).

Next, we examined the relationship between disease progression and the presence of pMAH135 genes based on VNTR genotyping. As shown in Fig. 1, pMAH135 genes were also conserved in cluster III strains, albeit with a few exceptions. The number of isolates with MAH_p47 in cluster III was significantly higher than the number of isolates without MAH_p47 ($p \leq 0.001$; Table 3), and a similar result was obtained for isolates with other pMAH135 genes (Fig. 1). Therefore, MATR-VNTR analysis showed that isolates
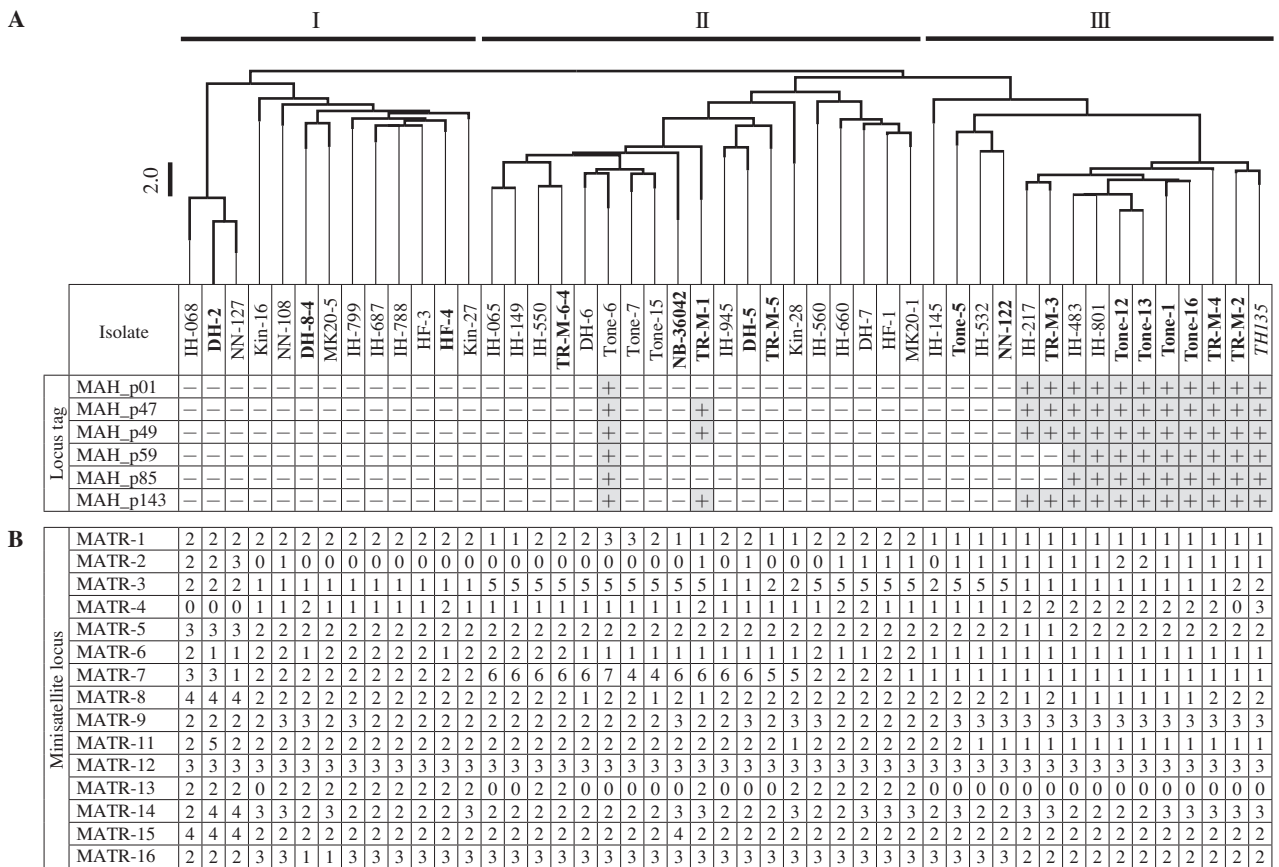
Fig. 1 *Mycobacterium avium* tandem repeats–variable number tandem repeat (MATR-VNTR) profiling of clinical isolates. (A) Dendrogram constructed from the results of MATR-VNTR typing analysis, showing the presence of pMAH135 genes for *M. avium* isolates, including 46 clinical strains (bold: strains from sputa of patients with deteriorated pulmonary disease; roman: strains from sputa of patients with stable pulmonary disease) and strain TH135 (italic). The dendrogram was created from distance matrix files by Fitch-Margoliash analysis according to MATR-VNTR markers. Scale bar indicates Manhattan distance. *M. avium* isolates were classified into clusters I to III by MATR-VNTR typing analysis. +, positive; −, negative. (B) MATR-VNTR typing analysis of *M. avium* isolates. The numbers of tandem repeat units at 15 minisatellite loci are shown for each *M. avium* isolates.

A — Dendrogram with clusters I, II, III. Scale bar = 2.0 (Manhattan distance).

Locus tag (presence of pMAH135 genes)

Isolate	IH-068	DH-2	NN-127	Kin-16	NN-108	DH-8-4	MK20-5	IH-799	IH-687	IH-788	HF-3	HF-4	Kin-27	IH-065	IH-149	IH-550	TR-M-6-4	DH-6	Tone-6	Tone-7	Tone-15	NB-36042	TR-M-1	IH-945	DH-5	TR-M-5	Kin-28	IH-560	IH-660	DH-7	HF-1	MK20-1	IH-145	Tone-5	IH-532	NN-122	IH-217	TR-M-3	IH-483	IH-801	Tone-12	Tone-13	Tone-1	Tone-16	TR-M-4	TR-M-2	*TH135*
MAH_p01	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	+	+	+	+	+	+	+	+
MAH_p47	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	+	+	+	+	+	+	+	+
MAH_p49	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	+	+	+	+	+	+	+	+
MAH_p59	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	+	+	+	+	+	+	+	+
MAH_p85	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	+	+	+	+	+	+	+	+
MAH_p143	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	+	+	+	+	+	+	+	+

B — Minisatellite locus (number of tandem repeat units)

Locus	IH-068	DH-2	NN-127	Kin-16	NN-108	DH-8-4	MK20-5	IH-799	IH-687	IH-788	HF-3	HF-4	Kin-27	IH-065	IH-149	IH-550	TR-M-6-4	DH-6	Tone-6	Tone-7	Tone-15	NB-36042	TR-M-1	IH-945	DH-5	TR-M-5	Kin-28	IH-560	IH-660	DH-7	HF-1	MK20-1	IH-145	Tone-5	IH-532	NN-122	IH-217	TR-M-3	IH-483	IH-801	Tone-12	Tone-13	Tone-1	Tone-16	TR-M-4	TR-M-2	*TH135*
MATR-1	2	2	2	2	2	2	2	2	2	2	2	2	1	1	2	2	3	3	2	1	2	1	1	2	2	1	1	2	2	2	2	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
MATR-2	2	2	3	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	1	1	1	1	0	1	1	1	1	1	1	2	2	1	1	1
MATR-3	2	2	2	1	1	1	1	1	1	1	1	1	1	5	5	5	5	5	5	5	5	5	5	1	1	2	2	5	5	5	5	2	5	5	5	1	1	1	1	1	1	1	1	1	1	2	2
MATR-4	0	0	0	1	1	2	1	1	1	1	1	2	1	1	1	1	1	1	1	1	1	2	1	1	1	1	2	1	1	1	1	1	2	2	2	2	2	2	2	2	2	2	2	2	0	3	3
MATR-5	3	3	3	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	1	1	1	2	2	2	2	2	2	2	2	2	2	2	2	2
MATR-6	2	1	1	2	2	1	2	2	2	2	1	2	2	2	2	1	1	1	1	1	1	1	1	2	1	1	2	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
MATR-7	3	3	1	2	2	2	2	2	2	2	2	2	2	6	6	6	6	6	7	4	4	6	6	6	6	5	5	2	2	2	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
MATR-8	4	4	4	2	2	2	2	2	2	2	2	2	2	1	2	1	2	1	2	1	2	2	1	2	2	2	2	2	2	2	2	1	2	1	2	1	1	1	1	1	1	1	1	2	2	2	2
MATR-9	2	2	2	3	3	2	3	2	2	2	2	2	2	2	2	2	2	2	2	2	3	2	2	3	2	3	3	3	2	2	2	2	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
MATR-11	2	5	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	1	2	2	2	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
MATR-12	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
MATR-13	2	2	2	0	2	2	2	2	2	2	2	0	0	2	2	0	0	0	0	0	2	0	0	0	0	2	2	2	2	2	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
MATR-14	2	4	4	3	3	2	3	2	2	2	2	2	2	3	3	2	3	2	3	2	3	3	2	3	3	3	2	3	2	3	2	2	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
MATR-15	4	4	4	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	4	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
MATR-16	2	2	2	3	3	1	1	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	2	2	2	2	2	2	2	2	2	2	2

Table 3 Relationships between isolates with pMAH135 genes and isolates from deteriorated and stable pulmonary disease patients according to VNTR genotype

VNTR cluster	Deteriorated disease patients[a] (n=17)	Stable disease patients[b] (n=29)	P value	MAH_p47		P value
				Positive[c] (n=12)	Negative[d] (n=34)	
Cluster I (n=13)	3 (17.6%)	10 (34.5%)	0.315	0 (0%)	13 (38.2%)	0.011
Cluster II (n=19)	5 (29.4%)	14 (48.3%)	0.235	2 (16.7%)	17 (50.0%)	0.089
Cluster III (n=14)	9 (52.9%)	5 (17.2%)	0.019	10 (83.3%)	4 (11.8%)	≦0.001

VNTR, variable number tandem repeats
[a]Strains from the sputa of patients with deteriorated pulmonary disease
[b]Strains from the sputa of patients with stable pulmonary disease
[c]Strains with gene-encoding MAH_p47
[d]Strains without gene-encoding MAH_p47

carrying pMAH135 genes and isolates from patients with deteriorated disease are significantly grouped in cluster III, indicating that these strains have a similar VNTR genotype. Taken together with Table 2, these findings indicate an association between pMAH135 genes and the progression of pulmonary disease.

Discussion

The development and exacerbation of pulmonary NTM disease depend not only on host factors but also on bacterial factors. Maekura et al. reported that pulmonary disease patients with serotype 4 *M.avium* strains had significantly poorer prognosis than those with other serotypes[10]. In recent studies, VNTR typing analysis of *M.avium*, *M.abscessus*, and *M.massiliense* isolates from patients with NTM lung disease revealed that isolates from patients with progressive lung disease and those with stable lung disease are clustered differently[20)29)], indicating an association between the VNTR genotype and lung disease progression. These results suggest the involvement of bacterial factors in the progression of pulmonary NTM disease, but no specific bacterial factors have been identified. In this study, the screening of clinical isolates from patients with different symptoms for pMAH135 genes, and VNTR typing analysis revealed an association between pMAH135 and the progression of pulmonary *M.avium* disease.

The clinical course of patients with pulmonary NTM disease is diverse, with some patients being stable without any treatment and others having deteriorating symptoms despite drug therapy, leading to severe lung damage[8)9)]. The relationship between the VNTR genotype and the progression of pulmonary *M.avium* disease shown in this and previous studies[20)] suggests that MATR-VNTR analysis is able to predict the progression of pulmonary *M.avium* disease. Furthermore, the findings in this study showed the possibility that it is much easier to predict the progression of pulmonary *M.avium* disease by examining the presence of pMAH135 genes than by performing MATR-VNTR analysis because of the strong correlation between the presence of pMAH135 genes and the VNTR genotype which is associated with the progression of pulmonary *M.avium* disease (Fig. 1, Table 3).
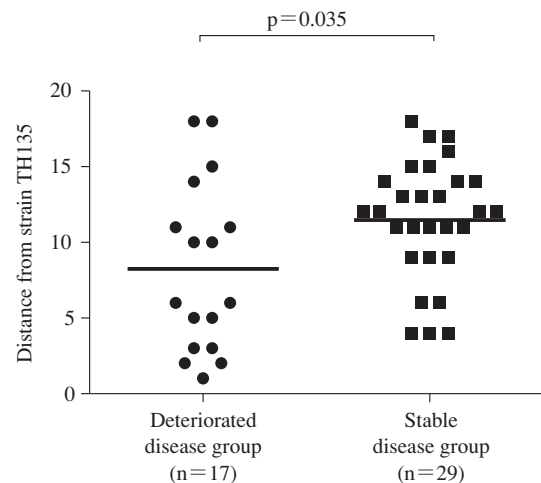

Fig. 2 Relationships between VNTR genotype and disease progression in pulmonary *M.avium* disease. The genetic distance was calculated as the Manhattan distance of *M. avium* isolates from the reference strain TH135. The results are shown for *M.avium* isolates from subjects with deteriorated or stable pulmonary *M.avium* disease. Horizontal lines indicate the mean value of genetic distance for the group; P values were calculated using a Mann-Whitney non-parametric comparison test.

However, to statistically reveal the association between pMAH135 and the progression of pulmonary *M.avium* disease and the association between pMAH135 and VNTR genotype in the progression of pulmonary *M.avium* disease, it is necessary to investigate the presence of pMAH135 in addition to pMAH135 genes by increasing the number of patients.

Treatment of pulmonary *M.avium* disease includes drug therapy and surgery. Although clarithromycin-based multidrug therapy is recommended for pulmonary *M.avium* disease, it is associated with two issues. Firstly, there are possible adverse effects of long-term treatment (12−24 months) and of drug toxicity in patients, and secondly, the efficacy of the drug therapy above certain levels is unknown[17)30)31)]. Consequently, predicting the clinical course of pulmonary *M.avium* disease is a highly useful clinical indicator for determining the appropriate treatment approach. Furthermore, the timing of treatment initiation influences outcomes and is thus considered

important. However, no clear criteria for determining the timing of treatment are currently available. The results of this study suggest that the presence of pMAH135 genes may serve as one criterion. The present study contains a number of limitations. First, it is possible that criteria for determining the timing of treatment varied among institutions. In addition, the ratios between the deteriorated and stable disease groups and the detection rates of pMAH135 genes in clinical isolates varied between institutions, necessitating further investigation. Comparison and analysis of the whole genome in clinical isolates to identify specific bacterial factors in strains from patients with worsened disease may not only clarify the role of pMAH135 as an exacerbating factor, but also reveal other bacterial factors involved in disease exacerbation.

In conclusion, the progression of pulmonary disease due to *M.avium* is associated with specific VNTR genotypes in *M.avium*. In addition, this study indicates an association between pulmonary disease progression and pMAH135, which codes for genes associated with *M.avium* pathogenicity and resistance to antimicrobial agents. This indicates the significance of bacterial factors in the disease progression. Taken together, these results indicate that the presence of pMAH135 genes might be a useful prognostic indicator for pulmonary *M.avium* disease and a criterion for determining the timing of treatment, suggesting the clinical utility of pMAH135 genes.

Conflict of interest

The authors declare that there is no conflict of interest related to this article.

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